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Loss of single immunoglobulin interleukin-1 receptor-related molecule leads to enhanced colonic polyposis in *Apc^{min}* mice

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Abstract

Background & Aims—Commensal bacteria can activate signaling by the toll-like and interleukin-1 receptors (TLR and IL-1R) to mediate pathogenesis of inflammatory bowel diseases and colitis-associated cancer. We investigated the role of the single immunoglobulin IL-1 receptor-related (SIGIRR) molecule, a negative regulator of TLR and IL-1R signaling, as a tumor suppressor to determine whether SIGIRR controls cell cycle progression, genetic instability, and colon tumor initiation by modulating commensal TLR signaling in the gastrointestinal tract.

Methods—We analyzed *Apc^{min/+}/Sigirr^{-/-}* mice for polyps, microadenomas, and anaphase bridge index. Commensal bacteria were depleted from mice with antibiotics. Akt, mTOR and β -catenin pathways were examined by immunoblotting and immunohistochemistry. Loss of heterozygosity (LOH) of *Apc* and expression of cytokines and proinflammatory mediators were measured by non-quantitative or quantitative PCR.

Results—*Apc^{min/+}/Sigirr^{-/-}* mice had increased LOH of *Apc* and microadenoma formation, resulting in spontaneous colonic polyposis, compared with *Apc^{min/+}/Sigirr^{+/+}* mice. The increased colonic tumorigenesis that occurred in the *Apc^{min/+}/Sigirr^{-/-}* mice depended on the presence of commensal bacteria in the gastrointestinal tract. Cell proliferation and chromosomal instability increased in colon crypt cells of the *Apc^{min/+}/Sigirr^{-/-}* mice. Akt, mTOR and their substrates were hyper-activated in colon epithelium of *Apc^{min/+}/Sigirr^{-/-}* mice in response to TLR or IL-1R ligands. Inhibition of the mTOR pathway by rapamycin reduced formation of microadenomas and polyps in the *Apc^{min/+}/Sigirr^{-/-}* mice.

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There is no conflict of interest to disclosure for all the authors.

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Conclusions—SIGIRR acts as a tumor suppressor in the colon by inhibiting TLR-induced, mTOR-mediated cell cycle progression and genetic instability.

Keywords

TIR8; Cyclin D1; c-Myc; Stat3

Introduction

The large and highly diverse populations of commensal bacteria in the mammalian colon are known to provide beneficial effects for the host, including the supply of nutrients and organic substrates¹⁻⁴. An important prerequisite for this host-bacterial mutualism is the “microbial tolerance” of the intestinal epithelial layer, a physical and immune barrier against commensal bacteria. Toll-like receptor (TLR) and Interleukin-1 receptor (IL-1R) signaling plays a critical role in the active cross-talk between the commensal-host, maintaining tolerance to the commensal bacteria. Inappropriate activation of the TLR-IL-1R signaling by commensal bacteria contributes to the pathogenesis of inflammatory bowel diseases and elevated risk for colorectal cancer⁵⁻¹⁰. Importantly, murine TLR4 deficiency leads to a marked reduction of colon tumor development in a colitis-associated cancer (CAC) model^{11, 12}, indicating the importance of TLR signaling in colon tumorigenesis. Therefore, the “microbial tolerance” is important for preventing both overt inflammation and tumorigenesis in the colon.

The “microbial tolerance” to commensal microflora is a consequence of multiple regulatory mechanisms, including modulation of TLR signaling^{11, 13, 14}. We and others previously reported that the Single Immunoglobulin IL-1 Receptor Related molecule (SIGIRR, also known as TIR8), a negative regulator for TLR-IL-1R signaling, plays a critical role in gut homeostasis, intestinal inflammation and colitis-associated tumorigenesis¹⁵⁻²¹. Tumorigenesis can be divided into three mechanistic stages, initiation, promotion and progression^{22, 23}. Using the colitis-associated cancer model, we have previously reported that SIGIRR deficiency increased inflammation-induced tumor promotion/progression. However, it remains unclear whether and how TLR signaling, in the absence of SIGIRR, impact on colon epithelium to enhance tumor initiation.

Aberrant activation of Wnt signaling represents the key initiating event for intestinal tumorigenesis, since activation of Wnt target genes dominantly drives proliferation of intestinal epithelium²⁴. Phosphorylation of β -catenin by glycogen synthase kinase 3 β (GSK3 β) leads to ubiquitination and degradation of β -catenin. Activation of Wnt signaling blocks the function of the “destruction complex” consisting of tumor suppressor Adenomatous polyposis coli (Apc), adaptor AXIN and GSK3 β , resulting in stabilization and nuclear accumulation of β -catenin²⁵. Constitutive activation of Wnt signaling is directly linked to colorectal cancer. Human genetic studies show that *Apc* is mutated in more than 95% of the Familial Adenomatous Polyposis (FAP) patients, and more than 80% of the sporadic colon cancers²⁶. Furthermore, 50% of colon carcinomas that retain wild-type *Apc*, acquire point mutations in β -catenin²⁷. *Apc*^{min/+} mice (containing a germ-line mutation in *Apc*) spontaneously develop a large number of tumors in small bowel, but very few polyps in the colon²⁸. Loss of heterozygosity (LOH) of *Apc*, a genetic alteration associated with the human colon cancers, is the exclusive genetic alteration leading to the tumor initiation in *Apc*^{min/+} mouse^{29, 30}. Thus, *Apc*^{min/+} mouse has been utilized as a spontaneous colon cancer model to identify genetic elements that have impact on chromosomal instability, resulting in LOH of *Apc* and tumor initiation³¹.

In this study, we introduced SIGIRR deficiency into the *Apc^{min/+}* mice and uncovered a critical role of SIGIRR in tumor initiation for the first time. SIGIRR deficiency leads to increased LOH of *Apc* and microadenoma formation, resulting in spontaneous colonic polyposis in *Apc^{min/+}/Sigirr^{-/-}* mice. Importantly, we found that mTOR and its downstream substrates, well-known regulators for translational control, were hyper-activated in SIGIRR-deficient colon epithelium in response to TLR-IL-1R ligands. Furthermore, the abrogation of mTOR pathway by rapamycin results in decreased microadenoma and polyps formation in *Apc^{min/+}/Sigirr^{-/-}* mice. Moreover, the increased colonic tumorigenesis in *Apc^{min/+}/Sigirr^{-/-}* mice is dependent on the presence of commensal bacteria in the gut, underscoring the role of dysregulated commensal bacteria-TLR signaling in colonic tumor initiation.

Materials and Methods

Mice Breeding and Genotyping

Apc^{min/+} mice on C57BL/6 background were purchased from Jackson Lab (Bar Harbor, ME) and bred onto *Sigirr^{-/-}* mice (>10 generations on C57BL/6 background) to generate *Apc^{min/+}/Sigirr^{+/-}* and *Sigirr^{+/-}* mice. *Apc^{min/+}/Sigirr^{+/-}* males were then crossed to *Sigirr^{+/-}* females, generating littermates including *Apc^{min/+}/Sigirr^{+/+}*, *Apc^{min/+}/Sigirr^{+/-}*, and *Apc^{min/+}/Sigirr^{-/-}* mice. These mice were bred and maintained in a pathogen-free animal facility at Cleveland Clinic Foundation. All the procedures were conducted in compliance with a protocol approved by the Institutional Animal Care and Use Committee at Cleveland Clinic Foundation. Genotyping of wild type and *Apc^{min}* alleles was conducted by PCR following a protocol provided by Jackson Laboratory. Genotyping of wild type and mutated *Sigirr* alleles was done by southern blotting as described previously¹⁸. All the procedures have been approved by the Institutional Animal Care and Use Committee at Cleveland Clinic Foundation.

Tumor Counts and Histology

Apc^{min/+}/Sigirr^{+/+}, *Apc^{min/+}/Sigirr^{+/-}*, and *Apc^{min/+}/Sigirr^{-/-}* littermates were euthanized at 3-4 months of age or when the first signs of morbidity appeared. The intestinal tract was removed immediately after the euthanasia, opened longitudinally and washed with PBS. The small intestine and colon were examined and tumor were counted and measured under a stereomicroscope. Colonic tissues and tumors were fixed by 10% buffered formalin overnight, and then embedded in paraffin. Paraffin-embedded specimens were cut to 5 μ m sections and stained with H&E for histology analyses.

Western Blotting

Purified primary colonocytes (treated or untreated) were lysed in cell lysis buffer (50mM Tris-HCl, pH 7.5/150mM NaCl/1% Triton X-100/1mM EDTA) supplemented by protease inhibitors Complete Mini (from Roch), and 1mM PMSF/2mM Na3VO4/5mM NaF. Protein concentration was measured by BCA assay kit from Sigma. 50 μ g total protein of each sample was resolved by 8-10% of SDS-PAGE, and transferred onto PVDF membrane. Western blotting was conducted using the following antibodies. Rabbit anti-p-mTOR, p-p70S6K, p-S6, p-Akt (S473), p-GSK3 α/β , p-4EBP, mTOR, p70S6K, S6, Akt, GSK3 α/β , 4EBP were purchased from Cell Signaling Technology, and used in 1:1000 dilution. Anti-c-Myc, p-cdk2, cdk2, cyclin E, cyclin A, cyclin D1 and actin were purchased from Santa Cruz, and diluted 1:500 for Western blotting.

Statistic analysis

ANOVA was used to analyze the tumor burden. Fisher's exact test was used to compare percentage. Student's T test was used for other studies and $p < 0.05$ is considered significant.

Results

SIGIRR deficiency leads to increased colon tumorigenesis in *Apc^{min/+}* mice

To investigate the role of SIGIRR in tumorigenesis in a spontaneous colon cancer model, *Sigirr^{-/-}* mice were bred onto the *Apc^{min/+}* background to generate *Apc^{min/+}/Sigirr^{+/+}*, *Apc^{min/+}/Sigirr^{-/-}* and *Apc^{min/+}/Sigirr^{+/-}* mice. Intestinal samples from littermates at 12-16 weeks of age were examined for tumor formation. As expected, *Apc^{min/+}/Sigirr^{+/+}* developed on average 42 tumors per small intestine, but only 1.3 tumors per colon on average (Fig. 1A). Importantly, *Apc^{min/+}/Sigirr^{-/-}* mice developed on average 4.5 polyps per colon (more than 3-fold increase as compared to *Apc^{min/+}/Sigirr^{+/+}* mice) (Fig. 1A and B), although these *Apc^{min/+}/Sigirr^{-/-}* mice only exhibited a modest increase in small intestinal polyps (average 67 polyps per mouse) (Fig. 1A). It is also noted that colon and small intestine polyps developed in *Apc^{min/+}/Sigirr^{+/-}* mice were comparable to those in *Apc^{min/+}/Sigirr^{+/+}* mice, implicating a haplosufficiency for SIGIRR function (Fig. 1A and B). Remarkably, about 60% polyps in *Apc^{min/+}/Sigirr^{-/-}* colon were larger than 2 mm in diameter, whereas only 36% polyps in *Apc^{min/+}/Sigirr^{+/+}* colon were big adenomas (>2mm in diameter) (Fig. 1C). Moreover, more large polyps (>4mm in diameter) were developed in *Apc^{min/+}/Sigirr^{-/-}* small intestine than *Apc^{min/+}/Sigirr^{+/+}* small intestine (Fig. 1C). Notably, the polyps developed in *Apc^{min/+}/Sigirr^{-/-}* mice were tubular adenomas with high degree of dysplasia (Fig. 1D).

The critical role of commensal bacteria in tumorigenesis

The elevated colonic tumorigenesis in *Apc^{min/+}/Sigirr^{-/-}* mice might be due to dysregulated TLR signaling triggered by commensal bacteria derived ligands. To test this hypothesis, commensal bacteria were depleted by oral administration of a cocktail of antibiotics¹⁵. Antibiotics treatment resulted in a significant reduction of tumor formation in the colon and small intestine of the *Apc^{min/+}/Sigirr^{-/-}* mice (Fig. 2A and B). In addition, the tumors developed in antibiotics treated *Apc^{min/+}/Sigirr^{-/-}* mice were considerably smaller than those in untreated controls (Fig. 2A). The expression of the signature TLR-responsive genes, such as KC, IL-6, and cyclin D1, were indeed substantially reduced in the mucosa and tumors of *Apc^{min/+}/Sigirr^{-/-}* after removal of commensal bacteria (Suppl. Fig. 1), suggesting an important link between commensal bacteria-induced TLR signaling and tumorigenesis in *Apc^{min/+}/Sigirr^{-/-}* colon. As a control, *Apc^{min/+}/Sigirr^{+/+}* mice were also treated with antibiotics. It is important to note that depletion of microflora only had a modest impact on the intestinal tumorigenesis in *Apc^{min/+}/Sigirr^{+/+}* mice (Fig. 2C). These results indicate that the elevated colonic tumorigenesis in *Apc^{min/+}/Sigirr^{-/-}* mice has a greater dependence on gut microflora and TLR signaling than the intestinal tumorigenesis in the *Apc^{min/+}/Sigirr^{+/+}* mice.

SIGIRR deficiency leads to increased tumor initiation in *Apc^{min/+}* mice

The key cellular events promoting tumor initiation include uncontrolled cell proliferation, prevention of apoptosis and elevated chromosomal instability. Consistent with the role of SIGIRR in colon epithelium homeostasis¹⁵, *Apc^{min/+}/Sigirr^{-/-}* mice manifested extended proliferation zones in their colonic mucosa, exhibiting elevated cell proliferation (*Apc^{min/+}/Sigirr^{-/-}*: 15.4±1.9 vs: *Apc^{min/+}/Sigirr^{+/+}*: 7.3±1.5 per crypt) (Fig. 3A-B), and concomitantly less apoptotic cells per crypt (*Apc^{min/+}/Sigirr^{-/-}*: 2.2±0.7 vs *Apc^{min/+}/Sigirr^{+/+}*: 3.3±0.8 per crypt) (Fig. 3C). In *Apc^{min/+}/Sigirr^{-/-}* tumors, three times more cells were undergoing active cell cycling (Fig. 3A and B) while fewer cells were eliminated by apoptosis (Fig. 3C). Taken together, increased cell proliferation and decreased apoptosis probably play a critical role in accelerating colon tumor development in *Apc^{min/+}/Sigirr^{-/-}* mice. These findings are consistent with the previous reports that demonstrate the ability of TLR signaling in promoting cell cycle progression^{32, 33}.

By examining the H&E stained sections from 6-weeks old mice, more microadenoma were detected in the colons and small intestines of *Apc^{min/+}/Sigirr^{-/-}* as compared to that of *Apc^{min/+}/Sigirr^{+/+}* mice (Fig. 4A and Suppl. Fig. 2), demonstrating increased tumor initiation in *Apc^{min/+}/Sigirr^{-/-}* mice. Loss of the wild-type *Apc* allele is the initial genetic alteration driving intestinal tumorigenesis in *Apc^{min/+}* mice^{31, 34}. Chromosomal instability attributable to LOH of *Apc* can be assessed by the frequency of “anaphase bridges”, extended chromosome bridging between the two spindle poles during the anaphase of cell cycle. By scoring anaphase bridges index (ABI) in the normal and tumor tissues, we detected significantly increased ABI in both normal mucosa and tumors of *Apc^{min/+}/Sigirr^{-/-}* mice compared to that in the *Apc^{min/+}/Sigirr^{+/+}* mice (Fig. 4B). In support of this, flow cytometry studies showed that DNA aneuploidy was significantly increased in *Apc^{min/+}/Sigirr^{-/-}* tumor cells compared to that in *Apc^{min/+}/Sigirr^{+/+}* mice (Suppl. Fig. 3).

It is plausible that the increased chromosomal instability in SIGIRR-deficient colon tissue might contribute to the increased tumorigenesis through LOH of *Apc* in *Apc^{min/+}/Sigirr^{-/-}* mice. By examining the *Apc* alleles in the normal and tumor tissues of the *Apc^{min/+}/Sigirr^{+/+}* and *Apc^{min/+}/Sigirr^{-/-}* colon, we found the wild-type *Apc* allele was lost in all the colon tumors dissected from *Apc^{min/+}/Sigirr^{+/+}* and *Apc^{min/+}/Sigirr^{-/-}* colons (Fig. 4C). In support of this, β -catenin became stabilized and accumulated in the cell cytoplasm and nucleus in microadenomas (Suppl. Fig. 4A) and well-developed tumors (Suppl. Fig. 4B). The fact that 100% of the tumors in *Apc^{min/+}/Sigirr^{+/+}* and *Apc^{min/+}/Sigirr^{-/-}* colons acquire LOH of *Apc* demonstrates a direct correlation between tumor incidence and LOH of *Apc*. Furthermore, more non-tumor colon epithelial cells in *Apc^{min/+}/Sigirr^{-/-}* mice showed cytoplasm and nuclear β -catenin staining (Suppl. Fig. 5), suggesting that *Apc^{min/+}/Sigirr^{-/-}* mice might have earlier and higher frequency of LOH of *Apc* in colon epithelial cells. Taken together, these results suggest that the increased chromosomal instability of the SIGIRR-deficient epithelium might contribute to a higher rate of LOH of *Apc* and consequent accumulation of β -catenin, promoting tumor initiation in *Apc^{min/+}/Sigirr^{-/-}* colon.

Hyper Akt-mTOR signaling contributes to spontaneous colonic polyposis in *Apc^{min/+}/Sigirr^{-/-}* mice

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that belongs to the phosphoinositide 3-kinase related kinase family. Akt-mTOR activated pathways have been linked to cell cycle progression and LOH of *Apc*, establishing Akt-mTOR signaling as a critical pathway driving tumor initiation in the *Apc^{min/+}* mouse³¹. Interestingly, we observed that SIGIRR deficiency resulted in increased constitutive activation of Akt and mTOR in the colon epithelial cells (Fig. 5A and Suppl. Fig. 6), implicating SIGIRR in the suppression of tumorigenesis through its impact on Akt-mTOR axis. Moreover, Akt and mTOR phosphorylation was further induced to a higher level by IL-1, CpG or LPS in *Apc^{min/+}/Sigirr^{-/-}* than that in *Apc^{min/+}/Sigirr^{+/+}* colon epithelial cells (Fig. 5A), suggesting that IL-1-TLR signaling probably contribute to the aberrant activation of the Akt-mTOR pathway in *Apc^{min/+}/Sigirr^{-/-}* colonic crypts.

Previous studies have shown that activation of the mTOR pathway promotes protein translation through the phosphorylation and inactivation of 4E-BP1 (a repressor for protein translation), and phosphorylation and activation of p70S6K³⁴. We found that 4E-BP1 phosphorylation was elevated in untreated or IL-1, CpG or LPS-treated *Apc^{min/+}/Sigirr^{-/-}* cells than that in *Apc^{min/+}/Sigirr^{+/+}* cells (Fig. 5A). Concomitantly, more phosphorylation of p70S6K and its substrate ribosomal protein S6 were detected in *Apc^{min/+}/Sigirr^{-/-}* cells compared to that in *Apc^{min/+}/Sigirr^{+/+}* cells untreated or treated by IL-1, CpG or LPS (Fig. 5A), confirming the hyper activation of the Akt-mTOR pathway in *Apc^{min/+}/Sigirr^{-/-}* colonic crypts. Moreover, the mTOR pathway was also constitutively activated in *Sigirr^{-/-}* colon epithelium and further elevated at a higher level upon IL-1 or LPS stimulation compared to

that in wild-type cells (Suppl. Fig. 7). These results indicate that SIGIRR critically controls mTOR activation in colonic epithelium by regulating IL-1 or commensal-TLR signaling.

The Akt-mTOR axis has been shown to promote cell cycle progression through its impact on posttranscriptional control of the key cell cycle regulators³⁴. Cyclin D1, cyclin E and c-Myc were indeed expressed at a higher level in *Apc^{min/+}/Sigirr^{-/-}* crypt cells compared to that in *Apc^{min/+}/Sigirr^{+/+}* cells (Fig. 5A). Furthermore, greater phosphorylation of cdk2 was detected in *Apc^{min/+}/Sigirr^{-/-}* crypt cells compared to that in *Apc^{min/+}/Sigirr^{+/+}* cells (Fig. 5A). *Apc^{min/+}/Sigirr^{-/-}* crypt cells also displayed decreased expression of cyclin-dependent kinase inhibitor p27, a negative regulator for G1-S transition (Fig. 5A). It is conceivable that dysregulation of these key cell cycle regulators might promote aberrant cell proliferation, contributing to the increased LOH of *Apc* and consequent more tumor initiation in *Apc^{min/+}/Sigirr^{-/-}* crypt cells.

Notably, Akt-mTOR signaling has also been shown to converge onto the Wnt- β -catenin pathway by phosphorylating GSK3 β , a key kinase in controlling the stability of β -catenin. GSK3 β phosphorylation was increased in both untreated and stimulated (by IL-1, CpG or LPS) *Apc^{min/+}/Sigirr^{-/-}* colon crypt cells compared to that in *Apc^{min/+}/Sigirr^{+/+}* cells (Fig. 5A). Interestingly, elevated GSK3 β phosphorylation was observed at the base of the crypts of *Apc^{min/+}/Sigirr^{-/-}* mice as compared to that in *Apc^{min/+}/Sigirr^{+/+}* mice (Suppl. Fig. 6). These results implicate that loss of SIGIRR might also directly impact on the β -catenin pathway through the Akt-mTOR-GSK3 β axis, contributing to the increased cell proliferation and LOH of *Apc* in *Apc^{min/+}/Sigirr^{-/-}* colon crypt cells.

Since SIGIRR-deficient epithelial cells displayed constitutive and hyper activation of mTOR in response to TLR-IL-1R ligands, we hypothesize that the Akt-mTOR activated pathway is the key molecular mechanism underlying the increased cell proliferation, LOH of *Apc*, and tumor initiation in *Apc^{min/+}/Sigirr^{-/-}* colonic epithelia. Rapamycin has been shown to specifically inhibit mTOR signaling and suppress intestinal polyp formation³⁵. We tested whether rapamycin is able to suppress tumor formation in *Apc^{min/+}/Sigirr^{-/-}* colon. The *Apc^{min/+}/Sigirr^{-/-}* mice were treated with rapamycin (5mg/kg, 5 times/week) by I.P. injection for two weeks for microadenoma formation. As expected, rapamycin treatment effectively inhibited mTOR pathway (Suppl. Fig. 8). Remarkably, cell proliferation, β -catenin activation, microadenoma formation were greatly inhibited in *Apc^{min/+}/Sigirr^{-/-}* mice by rapamycin (Fig. 5B and C, and Suppl. Fig. 9-10). Moreover, rapamycin treatment also resulted in reduced microadenoma formation in *Apc^{min/+}/Sigirr^{+/+}* mice, supporting a critical role for mTOR in driving tumor initiation in *Apc^{min/+}* mice (Suppl. Fig. 9-10). Furthermore, extended rapamycin treatment for 6 weeks substantially inhibited tumor growth in *Apc^{min/+}/Sigirr^{+/+}* and *Apc^{min/+}/Sigirr^{-/-}* mice (Suppl. Fig. 11). Taken together, these results confirm that mTOR signaling plays a critical role in spontaneous intestinal polyposis in *Apc^{min/+}/Sigirr^{-/-}* mice.

Increased inflammatory state within *Apc^{min/+}/Sigirr^{-/-}* colon tumors

The fact that SIGIRR deficiency leads to increased tumor incidence and the size of tumors suggests that SIGIRR-modulated signaling probably also impacts on tumor promotion/progression. NF κ B, a major component of TLR signaling, plays a critical role in tumor promotion by induction of proinflammatory signaling. In *Apc^{min/+}/Sigirr^{+/+}* epithelium, activated NF κ B staining was relatively weak, and exclusively induced in mature colonocytes at the top of crypts (Fig. 6A), which is consistent with a previous report documented NF κ B activation in colon crypts by *in vivo* imaging³⁶. In contrast, NF κ B activation was detected over the whole crypt axis, including differentiated and proliferating cells in *Apc^{min/+}/Sigirr^{-/-}* crypts (Fig. 6A).

Consistent with increased activation of NF κ B, a subset of NF κ B target genes were up-regulated in *Apc^{min/+}/Sigirr^{-/-}* colon. We detected much stronger induction of inflammatory gene COX2 and cytokines (IL-6 and IL-23) in both normal tissues and tumors of *Apc^{min/+}/Sigirr^{-/-}* mice (Fig. 6B). In particular, IL-6 has been shown to promote tumor progression in inflammation-associated cancer models through the activation of oncogene STAT3²³. Interestingly, STAT3 was predominantly activated at the base of the crypts, and its activation was greatly elevated in *Apc^{min/+}/Sigirr^{-/-}* mice (Fig. 6A). Consistently, cyclin D1, Bcl-xL and c-myc, which have been shown to be induced by NF κ B and STAT3, were up-regulated at a higher levels in the tumors of *Apc^{min/+}/Sigirr^{-/-}* mice (Fig. 6B), which might be responsible for the increased tumor progression in these mice.

The critical role of epithelial-derived SIGIRR in controlling colon tumorigenesis

Our results suggest that SIGIRR might provide a mechanism by which the normal intestinal epithelium guards against chronic activation and tumorigenesis in the presence of commensal flora. To test the role of epithelial-derived SIGIRR in controlling spontaneous intestinal tumorigenesis, we crossed gut epithelial specific SIGIRR-transgenic mouse (Sigirr IEC-TG, driven by Fabp promoter¹⁵) onto *Apc^{min/+}/Sigirr^{-/-}* background to generate *Apc^{min/+}/Sigirr^{-/-}/IEC-TG* mice, restoring SIGIRR expression only in intestinal epithelium. Compared to *Apc^{min/+}/Sigirr^{-/-}* mice, *Apc^{min/+}/Sigirr^{-/-}/IEC-TG* mice had fewer tumors in their colon and small intestine (Fig. 7). Moreover, the size of the tumors formed in *Apc^{min/+}/Sigirr^{-/-}/IEC-TG* mice were smaller than those in *Apc^{min/+}/Sigirr^{-/-}* mice, demonstrating SIGIRR-regulated TLR/IL-1R signaling in epithelium plays a critical role in tumorigenesis in the *Apc^{min/+}* model.

Discussion

In this manuscript, we report that SIGIRR is a critical tumor suppressor that controls colonic tumorigenesis by inhibiting TLR-induced mTOR-mediated cell cycle progression and consequent genetic instability. SIGIRR deficiency leads to increased LOH of *Apc* and microadenoma formation, resulting in spontaneous colonic polyposis in *Apc^{min/+}/Sigirr^{-/-}* mice. While Akt, mTOR and their downstream substrates are hyper-activated in *Apc^{min/+}/Sigirr^{-/-}* colon epithelium, the inhibition of mTOR pathway by rapamycin results in decreased microadenoma formation in *Apc^{min/+}/Sigirr^{-/-}* mice. Importantly, the spontaneous colonic polyposis in *Apc^{min/+}/Sigirr^{-/-}* mice is dependent on the presence of commensal bacteria in the gut, implicating the critical role of TLR signaling in colonic tumorigenesis in these mice.

We have previously used colitis-associated cancer (CAC) model to assess the role of SIGIRR in inflammation-induced tumorigenesis¹⁵. Using the CAC model, we reported that SIGIRR deficiency increased tumor promotion and progression. Due to lack of specific molecular markers, we failed to determine whether SIGIRR deficiency had direct impact on tumor initiation. The *Apc^{min/+}* model is based on a defined mutation in the tumor suppressor *Apc* gene and does not involve chronic inflammation. Importantly, the *Apc^{min/+}* model is a spontaneous colon cancer model mimicking the FAP syndrome, and LOH of *Apc* is the exclusive genetic alteration leading to the tumor initiation in *Apc^{min/+}* mouse^{29, 30, 37}. Therefore, introduction of a second mutation into the *Apc^{min/+}* mice allows us to determine whether this particular genetic alteration has any impact on LOH of *Apc*, which is the key initiation event in intestinal tumorigenesis.

The *Apc^{min/+}* mouse model helped to define for the first time about the critical role of SIGIRR in tumor initiation. LOH of *Apc* was detected in all the polyps, indicating that SIGIRR deficiency increased tumor initiation in *Apc^{min/+}/Sigirr^{-/-}* mice through its impact on LOH of *Apc* in epithelium. Consistent with this, microadenomas were developed at a

higher rate in *Apc^{min/+}/Sigirr^{-/-}* mice, and displayed constitutive β -catenin activation. Chromosomal instability, a process directly linked to tumor initiation by promoting LOH of *Apc*, can occur in cells with increased cell proliferation due to dysregulated cell cycle^{31, 38}. The anaphase-bridge index, an aberrant event commonly linked to genetic instability, was indeed enhanced in *Apc^{min/+}/Sigirr^{-/-}* colon epithelium. Therefore, SIGIRR can directly impact on genetic instability and tumor initiation in colon epithelium by regulating TLR-IL-1R signaling.

Another important finding of this study is the discovery of the critical molecular mechanism for SIGIRR-modulated TLR-mediated tumor initiation. SIGIRR deficiency leads to hyper TLR-IL-1R-mediated Akt-mTOR signaling, a critical pathway for cell cycle progression, LOH of *Apc* and consequent tumor initiation in the *Apc^{min/+}* mouse. Recent studies have demonstrated that the Akt-mTOR pathway increases protein translation of the key cell cycle regulators, by mediating the phosphorylation of a variety of translational initiation regulatory proteins, such as 4EBP and eIF4G, directly or indirectly through p70S6K^{34, 39}. Elevated expression of cyclin D1, cyclin E and c-myc was indeed observed in *Apc^{min/+}/Sigirr^{-/-}* crypt cells. Therefore, hyper-activation of mTOR and p70S6K in *Apc^{min/+}/Sigirr^{-/-}* colonocytes might be responsible for increased cell proliferation and higher LOH of *Apc* in *Apc^{min/+}/Sigirr^{-/-}* colon. Consistently, the abrogation of mTOR pathway by rapamycin resulted in decreased cell proliferation, less microadenoma formation in *Apc^{min/+}/Sigirr^{-/-}* mice. It is important to point out that intestinal microadenoma formation in *Apc^{min/+}/Sigirr^{+/+}* was also inhibited by rapamycin, demonstrating that mTOR is a key signaling pathway required for intestinal tumorigenesis in *Apc^{min/+}* mice. However, it is intriguing that depletion of microflora only had minimum impact on the intestinal tumorigenesis in *Apc^{min/+}/Sigirr^{+/+}* mice, whereas antibiotic treatment greatly diminished intestinal tumorigenesis in *Apc^{min/+}/Sigirr^{-/-}* mice. These results indicate that the intestinal tumorigenesis in *Apc^{min/+}/Sigirr^{-/-}*, but not in *Apc^{min/+}/Sigirr^{+/+}* mice, is dependent on gut microflora and TLR signaling. While it remains unclear how mTOR is required for intestinal tumorigenesis in *Apc^{min/+}/Sigirr^{+/+}* mice, our results suggest that SIGIRR suppresses intestinal tumorigenesis at least in part through its impact on TLR-induced mTOR activation. Collectively, we identified mTOR for the first time for its role in linking TLR-IL-1R signaling to tumor initiation.

Dysregulated mTOR pathway has been linked to a broad range of human cancers, including familial and sporadic colorectal cancers⁴⁰. Furthermore, several upstream regulators of mTOR, such as PTEN, LKB1 and TSC1/2, are genetically mutated and functionally associated with inherited cancer syndromes such as Cowden Syndrome, Peutz-Jeghers Syndrome and Hamman-Rich syndrome. Tuberous Sclerosis Complex^{39, 41}. Consistent with the effect of rapamycin in *Apc^{min/+}/Sigirr^{-/-}*, rapamycin and its analogues have been recently approved by FDA to treat advanced renal cell carcinoma, and shown promising signs in treating endometrial cancers and mantle cell lymphoma. Better understanding of mTOR regulation will be important for the development of this promising cancer therapy.

Taken together, our current study has clearly revealed a critical role for SIGIRR in suppressing tumor initiation in the gut epithelium. Interestingly, MyD88-deficiency on *Apc^{min/+}* resulted in impaired TLR-induced inflammation and tumor promotion, but not tumor initiation⁸, implicating MyD88 is dispensable in regulating tumor initiation in epithelium. While elevated NF κ B signaling and inflammation mediated by immune cells primarily contribute to tumor promotion/progression in *Apc^{min/+}/Sigirr^{-/-}* mice, this study suggests that SIGIRR-regulated TLR-IL-1R signaling in epithelial cells might directly impacts on tumor initiation. It is important to point out that re-expression of SIGIRR only in intestinal epithelium resulted in a modest, but significant reduction of tumorigenesis in *Apc^{min/+}/Sigirr^{-/-}* mice, suggesting the critical role of epithelial-derived SIGIRR in

modulating colon tumorigenesis. On the other hand, the $Apc^{min/+}/Sigirr^{-/-}$ IEC-TG mice still exhibited higher tumorigenesis than $Apc^{min/+}/Sigirr^{+/+}$ mice, suggesting that SIGIRR deficiency in other lineages, such as T cells, may also play a role in controlling tumorigenesis in $Apc^{min/+}/Sigirr^{-/-}$ mice as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. SIGIRR deficiency results in increased intestinal tumorigenesis on *Apc^{min/+}* background. (A) Tumor numbers detected in the colon and small intestine of *Apc^{min/+}/Sigirr^{+/+}*, *Apc^{min/+}/Sigirr^{+/-}*, and *Apc^{min/+}/Sigirr^{-/-}* littermates. (B) Macrophotograph of the representative colons of *Apc^{min/+}/Sigirr^{+/+}*, *Apc^{min/+}/Sigirr^{+/-}*, and *Apc^{min/+}/Sigirr^{-/-}* littermates. (C) Size distribution of the colon and small intestine tumors developed in *Apc^{min/+}/Sigirr^{+/+}* and *Apc^{min/+}/Sigirr^{-/-}* littermates. (D) Histology of colon tumors developed in *Apc^{min/+}/Sigirr^{+/+}* and *Apc^{min/+}/Sigirr^{-/-}* littermates. ANOVA (A) or Fisher's Exact Test (C) was used for statistic analysis. *= $p < 0.05$, **= $p < 0.01$, N.S. = Not significant.

Fig. 2.

Antibiotics-treatment ameliorates tumorigenesis in *Apc^{min/+}/Sigirr^{-/-}* mice. (A) The number and size distribution of colon tumors developed in *Apc^{min/+}/Sigirr^{-/-}* mice with or without antibiotics-treatment. (B) Small intestinal tumors developed in *Apc^{min/+}/Sigirr^{-/-}* and *Apc^{min/+}/Sigirr^{+/+}* mice with or without antibiotics-treatment. (C) The number and size distribution of colon tumors developed in *Apc^{min/+}/Sigirr^{+/+}* mice with or without antibiotics-treatment. *Apc^{min/+}/Sigirr^{+/+}* or *Apc^{min/+}/Sigirr^{-/-}* mice at 6 weeks of age were fed with regular water or water containing 1 g/L ampicillin, 500 mg/L vancomycin, 1 g/L neomycin and 1 g/L metronidazole for 6-8 weeks. ANOVA (A and B) or Fisher's Exact Test (C) was used for statistic analysis. *= $p < 0.05$, **= $p < 0.01$, N.S. = Not significant.

Fig. 3. Increased cell proliferation and decreased apoptosis in *Apc^{min/+}/Sigirr^{-/-}* colon. (A) Immunohistochemistry staining of Ki-67 in *Apc^{min/+}/Sigirr^{+/+}* and *Apc^{min/+}/Sigirr^{-/-}* colon. Magnification: 100× or 400×. (B) and (C) Quantification of Ki-67 positive cells (B) or apoptotic cells detected by *in situ* TUNEL assay (C) in normal crypts and tumors. Well-oriented crypts (at least 50 normal crypts), or representative tumor fields (at least 30 high power fields) were counted on 2-4 slides from each mouse and 3 pairs of mice were analyzed. Error bars represent $\pm$ SEM, and Student's T Test was conducted. * = $p < 0.05$, ** = $p < 0.01$.

Fig. 4. Increased tumor initiation in *Apc^{min/+}/Sigirr^{-/-}* colon. (A) microadenoma developed in *Apc^{min/+}/Sigirr^{+/+}* and *Apc^{min/+}/Sigirr^{-/-}* colon. Swiss-rolls of colon from 5 weeks old mice were cut into 5µM consecutive sections and stained by H&E. Microadenomas were quantified over H&E stained sections from 5 pairs of mice. (B) Anaphase-bridge index was counted on H&E stained normal mucosal and colon tumors. Representative photographs show normal nuclei separation in anaphase or abnormal anaphase bridge. Magnification: 1000×. More than 300 anaphase tumor cells and 500 anaphase normal cells from each strain were examined for anaphase-bridge formation. ABI was quantified over H&E stained sections from five pairs of mice. (C) LOH of *Apc* was assessed by mismatched PCR^{29, 30}. Amplified *Apc* alleles were digested by HindIII and then separated by 1.5% agarose gel. A 123-bp product from the *Apc*⁺ allele and a 144-bp product from the *Apc^{min}* allele were detected. LOH of *Apc* was detected in all the colon tumors isolated from both strains. In this study, 20 colon tumors from *Apc^{min/+}/Sigirr^{-/-}* mice (N=4) and 5 colon tumors from *Apc^{min/+}/Sigirr^{+/+}* mice (N=4) were analyzed, and the representative data is shown. Error bars represent ±SEM, and Student's T Test was conducted. * = p<0.05, **=p<0.01.

Fig. 5.

Elevated mTOR pathway critically regulates tumorigenesis in *Apc^{min/+}/Sigirr^{-/-}* mice. (A) Cell lysates (50 μ g per sample) made from primary colonocytes untreated or treated by IL-1, LPS, or CpG for indicated times, were probed by antibodies against Akt-mTOR pathway and cell cycle regulators. (B) Rapamycin treatment inhibits cell proliferation in the colon of *Apc^{min/+}/Sigirr^{-/-}* mice. Four weeks old mice were treated by rapamycin (Sigma, St. Louis) (5mg/kg, 5 I.P. injections per week) for two weeks. Representative Ki-67 staining images were shown. Magnification: 100 $\times$. Quantification of Ki-67-positive cells was conducted on colon sections from three treated and three untreated mice (more than 50 well-oriented crypts were counted). (C) Rapamycin treatment inhibits microadenoma formation. Four weeks old mice were treated by rapamycin (5mg/kg, 5 I.P. injections per week) for two weeks. Swiss-rolls of colon and small intestine from untreated or rapamycin-treated mice were cut into 5 μ M consecutive sections and stained by H&E. Microadenomas were quantified over H&E stained sections from untreated or rapamycin-treated *Apc^{min/+}/Sigirr^{-/-}* or *Apc^{min/+}/Sigirr^{+/+}* mice (N=5 per group). Error bars represent $\pm$ SEM, and Student's T Test was conducted. * = $p < 0.05$, ** = $p < 0.01$.

Fig. 6.

Elevated NF κ B activation and tumor-promoting inflammation in *Apc^{min/+}/Sigirr^{-/-}* mice. (A) Immunohistochemistry staining of p-p65 or p-STAT3 on colon sections from *Apc^{min/+}/Sigirr^{+/+}* and *Apc^{min/+}/Sigirr^{-/-}* mice. Representative images are shown based on similar results obtained from three pairs of mice. Magnification: 100 $\times$ or 400 $\times$. (B) Induction of gene expression was examined by real-time PCR. Normal mucosal and tumors were dissected from 4 months old mice as described above. Real-time PCR was conducted on RNA samples made from 5 normal crypts and 10 tumors (about 2mm in diameter) in each group. Student's T Test was conducted, and data was shown as mean $\pm$ SEM. * = $p < 0.05$, ** = $p < 0.01$.

Fig. 7. Epithelial-derived SIGIRR inhibits tumorigenesis in *Apc^{min/+};Sigirr^{-/-}* mice. Fabp-SIGIRR transgenic mouse (IEC-TG)¹⁵ was bred onto *Apc^{min/+};Sigirr^{-/-}* to generate *Apc^{min/+};Sigirr^{-/-}* and *Apc^{min/+};Sigirr^{-/-};IEC-TG* mice. Littermates at age of 4 month old were sacrificed to count tumors formed in colon and small intestine. ANOVA was used for statistic analysis. *= $p < 0.05$.